One-step generation of allogeneic CAR-T cells by simultaneous multiplex knockout and site-specific transgene integration with the Pin-point™ base editing platform.

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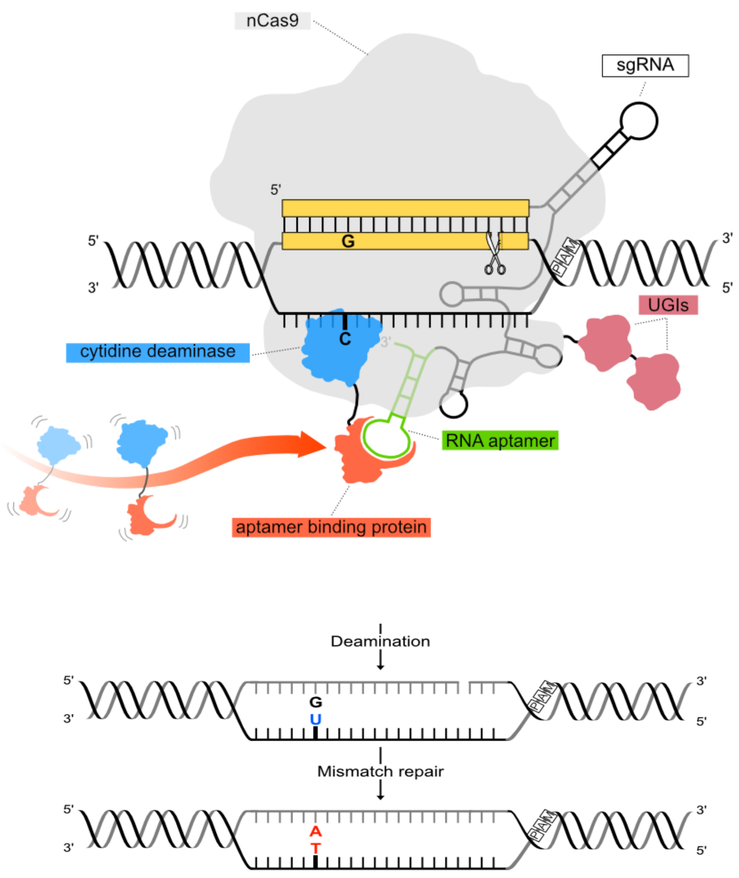
1 Abstract

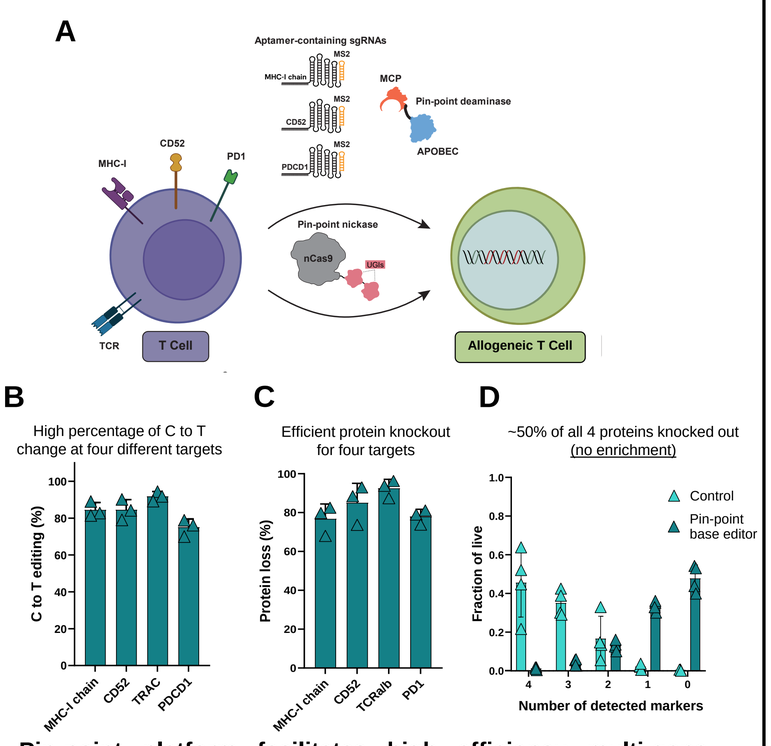
As our understanding of immune function regulators and the effects of the tumour microenvironment on immune cells improves, increasingly more complex cell engineering is being employed to improve the efficacy of cellular immunotherapies. We have developed the RNA aptamer-mediated Pin-point base editing platform with the aim to facilitate this complex cell therapy engineering in a safer manner than conventional nuclease-based technologies. The Pin-point base editing system is a modular technology where, in one configuration, it comprises a CRISPR-Cas and a deaminase module that can be delivered to the target cells as individual components. The assembly of the base editing machinery at the target locus relies on the interaction between an aptamer binding protein fused to the deaminase and an RNA aptamer located on the gRNA. The modularity and aptamer-dependent nature of the technology supports high flexibility in the customization of each individual component to address specific editing needs and enables complex genetic modifications. In this example, by combining aptamer-containing and aptamer-less gRNAs, we generated functional engineered CAR-T cells via simultaneous knockout of multiple targets by base editing alongside targeted chimeric antigen receptor (CAR) insertion at the endogenous TRAC locus. We used aptamer-less gRNAs to direct the nickase activity of the Cas enzyme to both DNA strands of the integration locus and stimulate homologous dependent repair (HDR) but avoiding the recruitment of the deaminase; whilst in the same transfection, aptamer-containing gRNAs recruit the deaminase to the other target sites for knockout generation by base editing. With this approach, site-specific knock-in and multiplex gene knockout are achieved within a single intervention and without the requirement to deliver additional sequence-targeting components, the introduction of multiple nuclease species, or more convoluted sequential editing strategies. We demonstrated high base editing efficiency and confirmed the safety of this approach by assessing the editing purity at all target sites, and carefully characterising the occurrence of DNA and RNA off targets, as well as genomic structural variants. The modularity and aptamer-dependent nature of the Pin-point base editing technology opens the possibility of specifically optimizing editing for each site in a different way and of combining multiple effectors to achieve advanced editing outcomes, broadening the applicability of this editing approach across oncology, autoimmunity, and the treatment of rare disease.

2 The modular Pin-point base editing platform



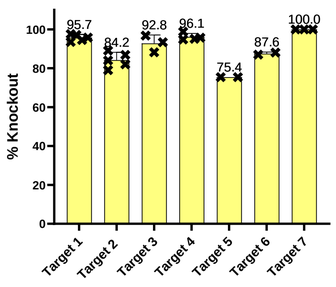
Schematic of one configuration of the modular Pin-point base editor system.
The Cas component is recruited to the DNA target sequence via a single guide RNA (sgRNA) encoding an aptamer in the scaffold region. The aptamer recruits a DNA-modifying deaminase to the DNA target sequence via an aptamer binding protein.
The Pin-point base editing platform allows precise genome modification by single nucleotide conversion. The platform is agnostic to the sequence targeting Cas, DNA modifying deaminase, and deaminase recruiting aptamer modules, and therefore provides the flexibility to assemble the most appropriate combination of elements for a given application.

3 Efficient multiplex editing in human T cells



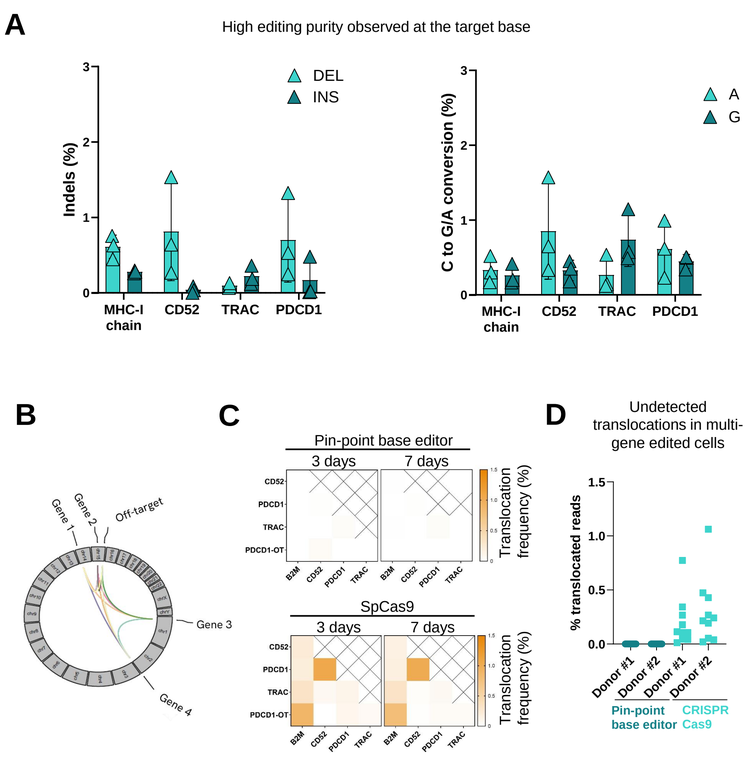
Pin-point platform facilitates high efficiency multi-gene editing in human T cells
A) Schematic of allogeneic T cell engineering via delivery of Pin-point nCas9-UGI and rAPOBEC1-MCP mRNAs, and synthetic aptameric sgRNAs targeting four genes to human T cells.
B) High levels of C to T editing achieved at each target in multi-gene edited T cells (NGS data).
C) High levels of protein knockout achieved for each target (flow cytometry).
D) Quantification of single, double, triple and quadruple negative target protein expression in edited or mick electroporated cells (flow cytometry).

4 Robust editing across multiple targets



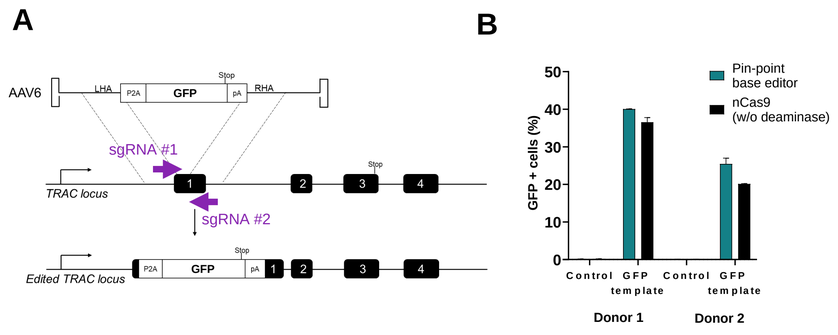
Pin-point platform can easily be adapted for efficient editing at multiple target sites
Pin-point nCas9 and rAPOBEC1-MCP mRNAs and individual synthetic aptameric gRNA were delivered to human T cells. Percentage of protein knockout reported using flow cytometry for targets 1-6 and by sequencing for target 7.

5 Precise editing and improved genome integrity



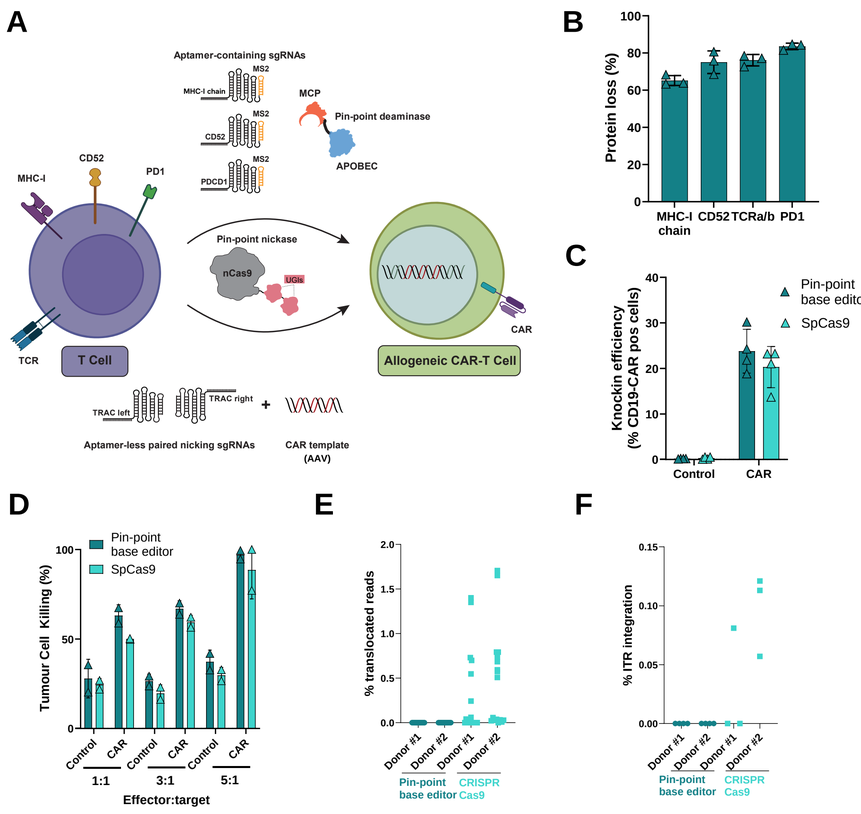
Precise editing outcome and reduced impact on genome stability
A) Low levels of unintended C to G or A conversion (left panel) and indel frequencies (right panel) at the target base in multi-gene edited T cells (NGS data).
B) Interchromosomal translocations identified in samples edited with SpCas9 at the four target sites.
C) Quantification of individual translocations in T cells edited at four targets with either the Pin-point platform or SpCas9 (ddPCR).
D) Undetectable translocations between the on- and off- target sites of the 4 gRNAs (MHC-I chain, CD52, TRAC, PDCD1) in T cells edited with the Pin-point base editor (targeted hybridisation-capture sequencing).

6 Targeted transgene knock-in compatible with the Pin-point platform



Pin-point platform facilitates locus specific knock-in.
A) Schematic of a GFP transgene knock-in using paired aptamer-less sgRNAs to target the nCas9-UGI component of the Pin-point base editor to the TRAC locus.
B) Knock-in efficiencies achieved with all base-editing components or with nCas9-UGI only for targeted integration of a promoter-less GFP transgene under the TRAC promoter.

7 Safer CAR-T cells generation by simultaneous knock-in and multiple knockouts



Pin-point platform facilitates multiplex base editing knockout and locus specific knock-in in one step engineering process.
A) Schematic of single-step CAR-T cells engineering by combining aptameric and aptamer-less sgRNAs to simultaneously knock-out multiple genes by base editing and to generate a CAR targeted transgene knock-in.
B) High levels of protein knockout achieved for each target (flow cytometry).
C) Comparable levels of transgene integration achieved by the Pin-point platform and SpCas9 (flow cytometry).
D) Killing activity of CAR-T cells generated with the Pin-point platform or SpCas9
E) Undetectable translocations between the on- and off- target sites of the 5 gRNAs in T cells edited via simultaneous knock-in and multiple knockouts with the Pin-point base editor (targeted hybridisation-capture sequencing).
E) Undetectable donor DNA integration at the sgRNA targeted sites (targeted hybridisation-capture sequencing).

8 Summary

- The aptamer-dependet Pin-point base editing platform facilitates efficient multi-gene editing in human T cells.
- Robustness of the platform is proven across multiple targets.
- Editing is precise at single bases and genome integrity is retained.
- Allogeneic CAR-T cells are obtained by one-step engineering via multiple base editing knockouts and simultaneous specific site CAR knock-in.
- The Pin-point platform overcomes the issues of translocations and donor DNA integration at off-target sites.

9 References

- Collantes et al. The CRISPR Journal, DOI:10.1089/crispr.2020.003
- Porreca et al. Mol Ther, DOI: 10.1016/j.ymthe.2024.06.033

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